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Characterization of Markers for the Identification and Isolation of Cancer Stem Cells

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CHARACTERIZATION OF MARKERS FOR THE IDENTIFICATION AND ISOLATION OF PROSTATE CANCER STEM CELLS

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ABSTRACT

Several models for prostate oncogenesis have been presented throughout the years, including the cancer stem cell (CSC) model. CSCs may be responsible for the self-renewing properties, and therefore progression, of the tumor. In this project, several protein and integrin markers were characterized using immunohistochemistry, immunofluorescence, and immunoblotting to identify cancer stem cells in human biopsy tissue. The data suggest that α2 and Trop2 cannot be used to identify CSCs, and that using several markers simultaneously, including perhaps CD133, will be required to identify, and eventually isolate, cancer stem cells.

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BACKGROUND

Cancer Stem Cells

Overview

Discovered roughly fifteen years ago in hematopoietic cancer (Regenbrecht *et al.*, 2008), cancer stem cells (CSCs) represent a small subset of cells in a tumor, and are different from the highly differentiated tumor cells (Morrison *et al.*, 2008). It is thought that CSCs arise from stem cells which undergo cancerous growth, while maintaining their ability to undergo differentiation like normal stem cells (Signoretti and Loda, 2007). It is also theorized that CSCs could be the product of the transformation of transit-amplifying cells (unipotent progenitor cells), or from the de-differentiation of adult cells that have acquired characteristics of stem cells (Signoretti and Loda, 2007). The cancer stem cell phenotype is defined by several distinct features that include self-renewal, differentiation, and extensive proliferation (Vaish, 2007). For cancer stem cells post-cell division, one of the daughter cells has identical genetic content to the parent cell and each stem cell is capable of differentiating into multiple lineages (Vaish, 2007). In recent years, cancer biologists have discovered that normal stem cells and cancer stem cells are similar, both phenotypically and functionally, as characterized in the hematopoietic system where hematopoetic stem cells (HSCs) and leukemic stem cells (LSCs) have been shown to express common cell surface markers (Yilmaz and Morrison, 2008). Similarities between these two cell types are also seen in their self-renewal pathways (Yilmaz and Morrison, 2008). This information is of great importance and relevance, as it promotes a clearer understanding of the self-renewal mechanism essential for tumor growth, allows for the identification of more specific

CSC markers, and reveals information about pathways that may be suitable as future treatments against cancer (Regenbrecht *et al.*, 2008).

Studies of cancer stem cells in solid tumors are still in the developmental stage (Regenbrecht *et al.*, 2008). Methods for the isolation of CSCs from solid tumors, as investigated in this MQP, have still yet to be established (Regenbrecht *et al.*, 2008). In cases where CSCs were successfully isolated from solid tumors, it is seen that they possess biological properties that directly correlate with patient prognosis (Regenbrecht *et al.*, 2008). In the brain tumor, it was reported that mechanisms of DNA damage repair are specifically activated in CSCs to confer resistance to radiation to them (Regenbrecht *et al.*, 2008). Thus, the identification and isolation of CSCs is the actual challenge for development of target therapies for cancer treatment (Regenbrecht *et al.*, 2008).

Cancer Stem Cell Model

Several models have been postulated to explain the origin and continued growth of tumors. The CSC hypothesis is derived from the fact that cancer cells do not form a homogenous population (Regenbrecht *et al.*, 2008). A heterogenous population of cancer cells could potentially result from the differentiation of a single stem cell. It is believed that cancer stem cells form a small portion of the tumor cell population (Regenbrecht *et al.*, 2008). Studies performed on these particular cells indicate that they are the only cells within the tumor population that are capable of maintaining tumor growth indefinitely (Regenbrecht *et al.*, 2008).

There are several possible origins of cancer stem cells. In the oldest theorized model (Lapidot *et al.*, 1994), CSCs emerge from normal stem cells by mutations that allow for an unchecked growth of CSCs (Lobo *et al.*, 2007). In newer models, however, it is viewed that

CSCs may arise from primary cancer cells that have acquired the ability to self-renew, occurring when several oncogenic mutations confer self-renewal capability onto regular cancer cells (Lobo *et al.*, 2007).

Evidence for the Existence of CSCs

Evidence for the existence of CSCs is provided by a mouse teratocarcinoma cancer model in which tumors arise from germinal totipotent stem cells. This model provides a way by which scientists are able to study how the cellular environment contributes to oncogenesis (Lobo *et al.*, 2007). Upon transplantation, these germinal stem cells form tumors consisting of both young and developed cell components, providing a model for how tumors form into a heterogeneous population of cells, and making this model useful for studying how the niche affects tumorigenesis (Lobo *et al.*, 2007).

Prostate Cancer

Cancer, a leading cause of death worldwide, causes one in every four deaths in the United States (Jemal *et al.*, 2009*)*. Prostate cancer is the most common non-skin cancer among men in United States (American Cancer Society, 2009), and is the second leading cause of death among men (Crawford, 2003). Almost 200,000 men in the country will be diagnosed with prostate cancer in 2010, leading to almost 28,000 deaths (Jemal *et al.*, 2009). Statistics show that 1 in 6 men will be diagnosed with prostate cancer at one point in their lives, with the likeliness increasing with age (Prostate Cancer Foundation, 2009). In fact, more than half of all prostate cancer cases are diagnoses of men 65 years and older (Prostate Cancer Foundation, 2009).

Despite these bleak numbers, the five-year survival rate for all stages of prostate cancer is near 100% (American Cancer Society, 2007).

Prostate cancer is thought to begin with a pre-cancerous state known as prostatic intraepithelial neoplasia (PIN) (American Cancer Society, 2009). PIN is seen in men as young as 20 years old, and by the time they reach 50, 50% of men show this condition (American Cancer Society, 2009). This condition shows physically noticeable changes in normal prostate gland cells, but at this point, abnormal cells would not be seen growing into other parts of the prostate, like cancerous cells (American Cancer Society, 2009).

While the majority of prostate tumors grow slowly, when they metastasize, they can do so very quickly (American Cancer Society, 2007). As such, prostate cancer often shows no early signs or symptoms (American Cancer Society, 2007). It is common for the prostate gland to become enlarged through the course of a man's life (National Kidney and Urologic Diseases Information Clearinghouse, 2006). This natural process is known as benign prostatic hyperplasia, BPH. Though having BPH does not increase the likelihood of developing prostate cancer, a man with BPH may have undetected prostate cancer as well, or may develop prostate cancer in the future (National Kidney and Urologic Diseases Information Clearinghouse, 2006). Since this enlargement process is natural and inevitable, age is the primary risk factor for prostate cancer (National Kidney and Urologic Diseases Information Clearinghouse, 2006).

The normal prostatic epithelium is composed of three different types of cells: secretory, basal, and neuroendocrine (Signoretti *et al.*, 2000). Cells have been identified in the normal prostatic epithelium which are a morphological and immunophenotypical intermediate between basal and secretory cells (Signoretti *et al.*, 2000). Evidence has been shown that basal and secretory cells are independent lineages and each have the ability to self-renew (Evans and

Chandler, 1987). Thus, the existence of a prostate stem cell able to give rise to both basal and secretory cells is controversial (Signoretti *et al.*, 2000).

Though the prostate is composed of several types of cells, the majority of prostate cancers develop from the luminal secretory cells (American Cancer Society, 2009). Other types of cancers that can also develop in the prostate are known as sarcomas, small cell carcinomas, and transitional cell carcinomas (American Cancer Society, 2009). However, since the other types are so rare, most prostate cancers can be assumed to be adenocarcinomas (cancers developed from luminal epithelium) (American Cancer Society, 2009). The human prostate cancer specimens evaluated in this MQP are adenocarcinomas.

Prostate Cancer Stem Cells

It has been postulated that prostate cancer arises from differentiated luminal cells, since most of the tumor cell population express luminal cell-specific markers, while being negative for basal markers (Lang *et al.*, 2009; Signoretti *et al.*, 2000). This information has also led to the hypothesis that prostate cancer arises from intermediate progenitor cells which have gained the ability via mutations to self-renew (Lang *et al.*, 2009). It has also been suggested that prostate CSCs arise from once normal stem cells (Lang *et al.*, 2009).

Integrins in Cancer

The integrin "superfamily" is composed of α and β heterodimeric transmembrane proteins that bind the extracellular matrix (ECM) to modulate cellular adhesion to substrate (Mizejewski, 1999). ECM proteins are essential for the normal development and remodeling of tissues during morphogenesis, tissue maintenance, wound healing, and oncogenesis (Mizejewski, 1999). Integrins are composed of long extracellular domains (see **Figure 1** for structure), which mediate adhesion to their ligands, and short cytoplasmic domains which bind/interact with several receptors to the cytoskeleton and intracellular signaling proteins involved in the regulation of signaling networks, such as cytoskeletal functions (Hayes *et al*., 2003).

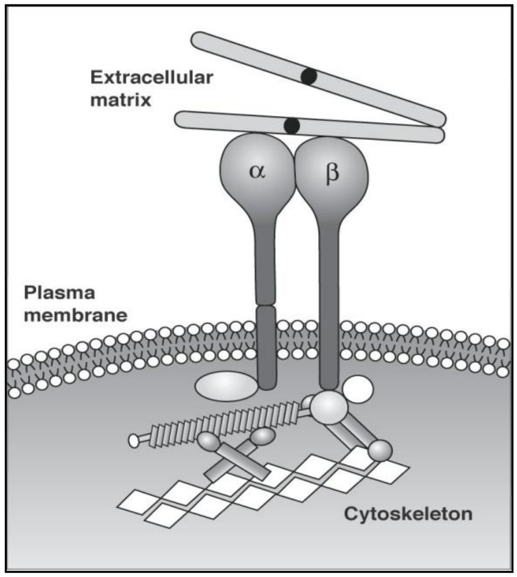


Figure 1. Diagram of Integrin Structure and Domains. Integrin receptors are composed of two subunits (α and β). The majority of amino acid residues are extracellular, and contain a variety of functional domains. (Goel *et al.,* 2004)

Integrin Deregulation in Prostate Cancer

In prostate cancer, tumor cells show abnormal expression of integrins and have a significantly different ECM, compared to normal prostatic cells. Integrin deregulation in cancer is thought to occur through activation of various specific transcriptional, translational and post-translational processes (Goel *et al.*, 2008).

Studies have reported that most α and β integrin subunits are downregulated as a prostate gland slowly turns cancerous. Specifically, several reports show that the α3, α4, α5, and α7 subunits are downregulated (Goel *et al.*, 2008). Integrin α2 is unique in that it is downregulated in prostate cancer while being upregulated in lymph node metastases, when compared to primary lesions (Goel *et al.,* 2008).

In contrast to most integrins, αvβ3, αvβ6, and a truncated version of α_{IIb} are upregulated in prostate cancer (Goel *et al,* 2008). In studies performed on two prostate epithelial cell lines, PC3 and LNCaP, it was seen that αvβ3 is expressed at high levels in PC3 as compared to LNCaP (Zheng *et al.,* 1999). PC3 cells are able to form metastatic lesions and intraperitoneal tumors, while LNCaP cells can form tumors but do not metastasize (Zheng *et al.,* 1999). This suggests a correlation between αvβ3 expression and metastatic potential of prostate cancer cells (**Figure 2**).

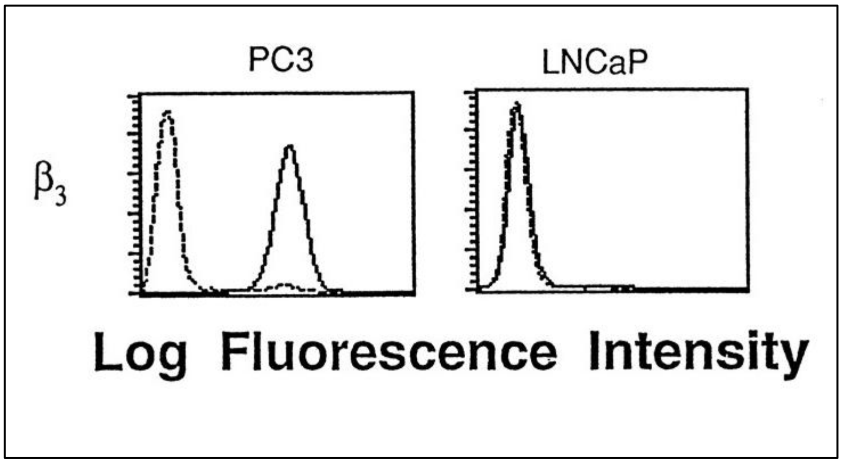


Figure 2. Overexpression of Integrin αvβ3 in Metastatic Prostate Cancer Cells. FACS analysis of αvβ3 integrin expression in two types of prostate cancer cell lines. PC3 cells show high metastatic potential, while LNCaP cells show low metastatic potential. Vertical axis represents cell number, while horizontal axis represents αvβ3 fluorescence expression and intensity. (Zheng *et al.,* 1999)

A summary of the disregulation of integrin α subunits in prostatic adenocarcinoma and metastases is shown in **Table I**.

Integrin disregulation during human prostate cancer progression			
α Subunit	Sample; Method	Deregulated Expression	References
α2	Tissue specimens; IHC	downregulated in adenocarcinoma*; upregulated in metastases	Nagle et al, 1994; Bonkhoff et al, 1993
α3, α4 α5	Tissue specimens; IHC	downregulated in adenocarcinoma	Nagle et al, 1994
α6	Tissue specimens; IHC, TEM	polarized distribution in benign, less polarized in HGPIN, not polarized in lymph node metastases; upregulated in metastases	Bonkhoff et al, 1993; Knox et al, 1994; Nagle et al, 1995
α7	Tissue specimens; IHC, Sequencing of genomic DNAs and cDNAs	downregulated in adenocarcinoma; also mutated in adenocarcinoma and recurrent adenocarcinoma	Ren et al, 2007
α‖b (truncated)	Tissue specimens; IHC	expressed in adenocarcinoma; absent in normal tissue	Trikha et al, 1998

Table 1. Integrin Disregulation During Human Prostate Cancer Progression.
(Goel *et al.,* 2008)

Transformation of a tumor cell from a benign state to a malignant one is characterized by the disruption of cytoskeletal organization, decreased cellular adhesion, and altered adhesion-dependent reactions (Mizejewski, 1999). The spatial arrangement of integrins becomes disordered, with a diffuse and sparse cellular distribution in carcinomas (Mizejewski, 1999). These cell-surface rearrangements can effect various integrin functions, such as ligand-binding affinity, and correlate with the disorganization of the membrane (Mizejewski, 1999). Abnormal interactions with the ECM induce cell proliferation, migration, differentiation, and overall cancer progression (Fornaro *et al.,* 2001).

Cancer Stem Cell Markers

Integrins as CSC Markers for Prostate Cancer

β3 Integrin

As previously described, β3 integrin, among other β subunits, is upregulated in human prostate cancer (Goel *et al.*, 2008). The β3 integrin subunit is known to localize in focal contacts, and also mediates the spreading and cytoskeletal rearrangement of normal, non-cancerous cells (Goel *et al.*, 2008). However, ectopic overexpression of β3 does not affect cell spreading (Zheng *et al.,* 1999), suggesting that the ability of β3 subunit to promote cancer progression is independent of on its effects on spreading (Goel *et al.*, 2008). It has also been concluded that the β3 subunit activates specific cell signaling pathways, and supports distinct cellular functions in cancer (*Goel et al.*, 2008). β3 has been shown to contribute to the establishment and growth of pulmonary metastatic melanoma lesions (Filardo *et al.,* 1995). This integrin has also been shown to increase the invasiveness of cutaneous melanomas from the epidermis to the dermis in studies performed by Hsu *et al* in 1998. Studies have also shown that β3 is necessary in cancer cells for increasing the levels of downstream effectors such as cdc2 (Manes *et al.,* 2003). Increased levels of cdc2 resulted in increased cell migration by its association with cyclin B2 and phosphorylation of caldesmon, a substrate of cdc2 (Manes *et al.*, 2003). Both caldesmon and cdc2 are localized in the membrane ruffles of motile cells, playing a key role in cancer cell migration (Manes *et al.,* 2003). Thus, increased β3 levels result in an increase of cdc2 levels, which in turn, promotes cancer cell migration (Manes *et al.,* 2003).

α2 Integrin

α2 integrin is expressed in the basal layer of the normal human prostate (Knox et al., 1994). However, its expression becomes aberrant in prostate cancer. In particular, it is downregulated in primary tumors and upregulated in lymph node metastases (Goel et al., 2008). α2 integrin has been demonstrated to be able to regenerate a fully differentiated prostate epithelium in immunocompromised mice (Gedye *et al.*, 2009). Moreover, cells which express both α2 and CD133 have a higher proliferative potential, and have a greater ability to reconstitute a normal prostate gland (Gedye *et al.*, 2009). These cells, showing expression of α2 and CD133, represent a tumor-initiating population within human prostate cancer (Maitland and Collins, 2008).

Other Markers for Prostate Cancer Stem Cells

p63 Basal Cell Marker

p63 is widely used for identification of normal prostate basal cells (Signoretti *et al.*, 2000). It is also selectively expressed in the basal cell compartment of a variety of other epithelial tissues (Signoretti *et al.*, 2000). Since this protein is undetectable in prostate cancer, its expression is used for the differential diagnosis between benign and malignant areas of the prostate gland (Signoretti *et al.*, 2000), making it a negative marker for prostate cancer.

CD133 Stem Cell Marker

CD133, also known as PROML1 or prominin, is known to be a stem cell surface antigen in several tissues, including the prostate (Choi *et al.*, 2009). It is a homologue of a mouse cell surface transmembrane glycoprotein, Prominin-1, and was originally found on neuroepithelial stem cells in mice (Miki *et al.,* 2007). It is used extensively to identify normal stem cells and cancer stem cells in a variety of tissues, such as hematopoietic, leukemic, neural, brain tumor, and most importantly, prostate cells (Miki *et al.*, 2007). Immunohistochemical studies have been performed to study localization of CD133 in the prostate, with the conclusion that CD133 may be unable to accurately identify cancer stem cells independently (Sullivan *et al.,* 2008). The distribution of CD133 across the prostate tissue is variable, and appears mostly in epithelial and stromal cell-specific, **Figure 3** (Sullivan *et al.*, 2008). However, since expression is seen in both benign and neoplastic tissue, (Figure 3), it was deduced that although CD133 may be a stem cell marker in the prostate, it may not be able to accurately identify cancer stem cells on its own (Sullivan *et al.,* 2008).

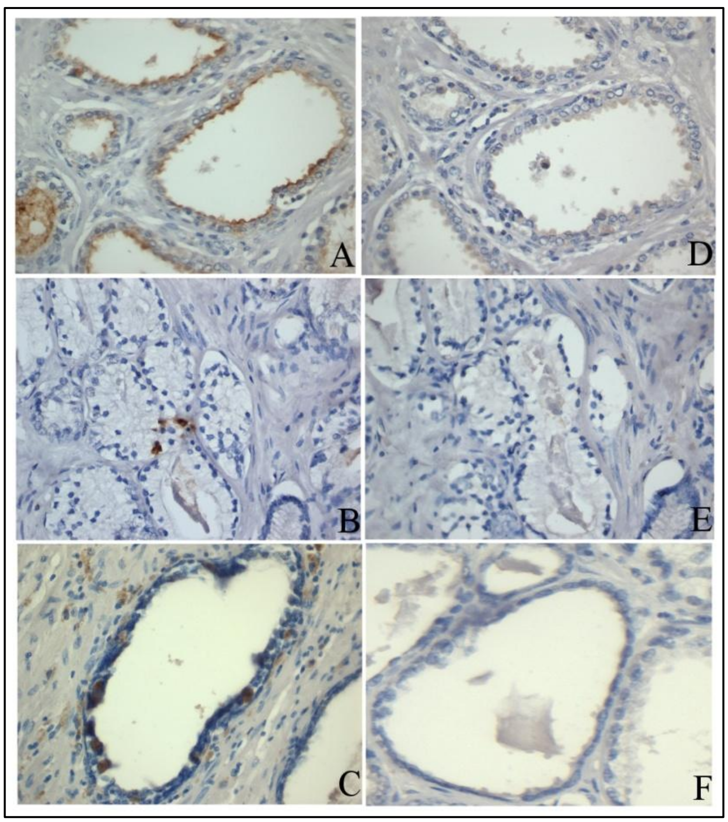


Figure 3. Expression of CD133 in Prostate Tissue. Immunohistochemistry for CD133 shows expression in neoplastic tissue (A) and benign glands (B and C). The respective negative controls, stained with rIgG, are seen in panels D-F. Expression is seen to be epithelial-cell specific and diffuse in epithelial and stromal cells (Sullivan *et al.,* 2008).

Trop2 Stem Cell Marker

Trop2 is a transmembrane tumor-associated calcium signal transducer whose function or ligand is not completely known, that is expressed in human epithelia, and frequently upregulated in carcinomas (Fornaro *et al.*, 1995; Ohmachi *et al.*, 2006; Fong *et al.*, 2008). Its expression has been reported in both normal and malignant tissue from organs such as kidney, lung, ovary, testes, etc (Goldstein *et al.*, 2008). Trop2 has been recently used to functionally discriminate between two basal cell subpopulations in the human prostate (Goldstein *et al.*, 2008). Though not all basal cells exhibit stem cell characteristics, basal cells expressing high levels of Trop2 are found to be enriched with *in vitro* and *in vivo* stem cell-like characteristics (Goldstein *et al.*, 2008). The role of Trop2, though not elucidated in normal and cancer cells, is suggested to be directly related to tumorigenicity and invasion of cancer cells (Goldstein *et al.*, 2008).

PROJECT PURPOSE

As discussed in the Background, understanding cancer stem cells is of utmost importance in the study and treatment of cancer. Several models have been presented throughout the years for prostate oncogenesis. The hypothesis being tested by this MQP is that the existence of an integrin marker for cancer stem cells can be confirmed by coexpression with known stem cell markers. Human prostate tissue specimens were analyzed by immunohistochemistry for stem cell marker Trop-2, as well as for integrins β3 and α2. Staining for prostate basal cell marker p63 was also performed for the identification of cancer cells which lack p63 expression. Double staining was performed for CD133 and α2 to identify potential cancer stem cell candidates. Immunofluorescence was performed to show localization of Trop-2 in prostate cancer cells, and immunoblotting was performed to analyze potential aberrations of Trop2 elecTrophoretic mobility. I hypothesize coexpression of integrin markers β3 (upregulated in prostate cancer) and α2 (upregulated in metastases) with stem cell markers CD133 and Trop-2 in regions negative for p63. This MQP study will identify novel cancer stem cell populations, which can be then isolated and characterized, for further studies for research and therapeutic purposes.

MATERIALS AND METHODS

Immunohistochemistry (IHC)

Paraffin-Embedded, Formalin Fixed Sections: (p63, and Trop2)

IHC staining was performed on 4µm sections prepared from paraffin-embedded blocks, placed on charged glass slides. The sections were deparaffinized in three changes of xylene for 10 minutes each. The slides were then hydrated in an ethanol series for 2 changes of 2 minutes each of 100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH, and dH_2O. The sections were then incubated in 3% hydrogen peroxide for 5 minutes to remove endogenous peroxidase activity. The slides were then blocked for non-specific staining. Next, the slides were incubated with the primary antibody. An IgG antibody raised in the same animal as the primary antibody was used as a negative control. After a series of 3 washes in PBS, slides were incubated with the primary antibody diluted in PBS+0.5% BSA. Slides were then washed 3 times with PBS + 0.05% Tween-20, and incubated with a biotinylated secondary IgG antibody. The conditions and durations for blocking, primary antibody, and secondary antibody incubation varied, and details can be seen in **Table 2**. Immunoperoxidase staining was performed using horseradish peroxidase streptavidin (Vector Labs, SA-5004), at a dilution of 1:100. The signal was amplified using the DAB Substrate-Chromogen System (Zymed Laboratories, Cat. #00-2014). Finally, the slides were counterstained with Mayer's hematoxylin, and dehydrated by reversing the alcohol and xylene series. Stained tissue sections were examined on an Olympus BX41 microscope and photographed using an Olympus DP12 camera. The IHC staining results were evaluated by Dr. Z. Jiang, Dr. L.R. Languino, Dr. H. L. Goel, Dr. M. Trerotola (University of Massachusetts

Medical School Departments of Pathology and Cancer Biology) and myself. The details for the cases used (Cases 1-15) can be seen in **Table 3**.

Frozen Sections: (CD133 and α2 Double Staining)

IHC staining was performed on 5μm sections prepared from frozen tumors, placed on charged glass slides. The sections were fixed in acetone at -20°C for 10 minutes. The slides were then blocked with 0.5% casein and 0.05% thimerosol diluted in TBS for 15 minutes at room temperature. The slides were then incubated with the primary antibody. An IgG antibody raised in the same animal as the primary antibody was substituted as a negative control. After a series of washes in PBS, slides were incubated with the primary antibody, diluted in PBS+0.1% BSA. Slides were then washed 3 times with PBS, and incubated with a biotinylated secondary IgG antibody. Immunoperoxidase staining was performed using horseradish peroxidase streptavidin (Vector Labs, SA-5004), at a dilution of 1:100. The signal was amplified using the DAB Substrate-Chromogen System (Zymed Laboratories, Cat. #00-2014). Finally, the slides were counterstained with Mayer's hematoxylin. The sections were then dehydrated in an ethanol series for 2 changes of 2 minutes each of 50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH, and Xylene. Stained tissue sections were examined on an Olympus BX41 microscope and photographed using an Olympus DP12 camera. The IHC staining results were evaluated by Dr. Z. Jiang, Dr. L.R. Languino, Dr. H. L. Goel, (University of Massachusetts Medical School Departments of Pathology and Cancer Biology) and myself. The cases used (Cases 16-21) did not have gleason grades available, but were graded by Dr. L.R. Languino and Dr. Z. Jiang.

Scoring of Expression

For all p63 IHC, expression was scored with a "+" and "-" system, indicating expression or lack of expression in benign and malignant regions. For all other IHC experiments, expression of the marker was seen in both benign and malignant regions. Thus, staining was scored with a "+", indicating the experiment was successful and yielded specific staining, or a "-", indicating that the experiment did not yield specific staining.

Double Staining

All double staining was performed using the Invitrogen PicTure Double Staining Kit (Cat No. 87-9999). The protocol followed was provided by the manufacturer, with aforementioned details for paraffin-embedded and frozen sections. α2 expression was seen by the brown HRP staining, and CD133 expression was seen by the red Fast-Red staining. Stained tissue sections were examined on an Olympus BX41 microscope and photographed using an Olympus DP12 camera. The IHC staining results were evaluated by Dr. Z. Jiang, Dr. L.R. Languino, Dr. H. L. Goel, Dr. M. Trerotola (University of Massachusetts Medical School Departments of Pathology and Cancer Biology) and myself. Areas in tissue sections showing both colors, or displaying double staining, were targeted as potential stem cells.

Immunofluorescence (IF)

Immunofluorescence was performed to examine the localization of Trop2 in PC3 cells transfected with Trop2, seeded on fibronectin. Cells were fixed on coverslips by incubation with 4% paraformaldehyde diluted in PBS for 5 minutes. To permeabilize the cells, coverslips were incubated with PBS+0.5% Triton for 5 minutes. The coverslips were then blocked with 5% BSA diluted in PBS for 20 minutes at room temperature. The coverslips were then incubated with the primary antibody (Trop2 T16) for 20 minutes, at a dilution of 1:80. After incubation with the primary antibody, the coverslips were washed several times with PBS. Coverslips were then incubated with the secondary FITC-conjugated goat anti-mouse antibody and Phalloidin-TRITC (1:1000, for Actin staining), in 5% BSA for 20 minutes. The coverslips were washed three times with PBS and mounted with anti fade reagent (Invitrogen, ProLong Gold antifade reagent, cat. P36930) for fluorescence microscopy (Olympus IX71).

Immunoblotting

Cells used for lysates were PC3-2 prostate cancer cells transfected with Trop2 or empty vector, for positive and negative controls, respectively. These cells do not endogenously express Trop2. Malignant and benign prostate tissue samples were also used for lysates. Cells and tissues were lysed in lysis buffer composed of 100 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 1% Triton X-100. Tissue samples were homogenized for 5 minutes until even suspension. The suspension was decanted, and an equal volume of 10% SDS was added to make final concentration of 5% SDS. The samples were mixed and boiled for 5 minutes. The tissue samples were then centrifuged at 14,000 RPM for 20 minutes at 4°C. The supernatants were then collected for BCA total protein assay.

Total cellular protein was resolved by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with mAb to Trop2. Proteins were separated by 10% SDS-PAGE under non reducing conditions, and immunoblotted with an Ab specific to FAK as a loading control.

Protein was visualized using an ECL reagent (Boston Bioproducts), and developed using autoradiography (ISC Bioexpress).

Table 3. Antibody and IHC Details

Marker	Antibody	Stock Concentration	Dilution	Blocking	Antigen Retrieval	Primary Incubation	Secondary Incubation	Reference
p63	Santa Cruz Biotechnology p63 [4A4]	200 μg/mL	1:50	Horse Serum, 20 minutes	10mM Sodium Citrate Buffer, pH 6.0 750W Microwave Oven, 15 minutes	4°C, overnight	Vector Labs, BA-2000 Biotinylated Anti-Mouse IgG (H+L), 30 minutes	Emanuel *et al*., 2004
CD133	Abcam Rb pAb to CD133 ab19898-100	1 mg/mL	1:400	Blocking solution (Invitrogen PicTure Kit), 30 minutes	-	4°C, overnight	Biotinylated Anti-Rabbit IgG Conjugated to Alkaline Phosphatase (Invitrogen PicTure Kit)	Friedman *et al*., 2009
Trop2	R&D Anti-Trop2 AF650	1 mg/mL	1:200	1% BSA, 5% Rabbit Serum in TBS, 1 hour	Microwave Oven, 8 minutes	1 hour, RT	Vector Labs, BA-9500 Biotinylated Anti-Goat IgG (H+L), 1 hour	Fong *et al.*, 2008
β3	Abcam, mAb to Integrin beta 3 [BV4] ab7167-50	100 μg/mL	1:50, 1:100	Horse Serum, 20 minutes	After pressure is reached in pressure cooker, 3 minutes	4°C, overnight	Vector Labs, BA-2000 Biotinylated Anti-Mouse IgG (H+L), 30 minutes	Neto *et al.*, 2007
	Hybridoma Culture Supernatant AP3		1:10, 1:5	Goat Serum, 45 minutes	Proteinase K, 37°C, 30 minutes	4°C, overnight	Hybridoma Culture Supernatant 12CA5, 45 minutes	Degrosellier *et al*., 2009
	Purified Antibody AP3	30 μg/mL, 10 μg/mL		Goat Serum, 45 minutes	Proteinase K, 37°C, 30 minutes	4°C, overnight	Hybridoma Culture Supernatant 12CA5, 45 minutes	Degrosellier *et al*., 2009
α2	Santa Cruz Biotechnology α2 [HAS-3]	200 μg/mL	1:10	TBS + 0.5% casein and 0.05% thimerosal, 15 minutes	-	4°C, overnight	Vector Labs, BA-2000 Biotinylated Anti-Mouse IgG (H+L), 30 minutes	Yoshimura *et al.*, 2009

Table 3. Paraffin-Embedded Formalin Fixed Case Details							
Case	**Tissue Type**	**Diagnosis**	**A/S/R**	**Necrosis %**	**Lesion %**	**Stroma %**	**Gleason Grade**
1	Malignant	Adenocarcinoma	74/M/W	0	70	30	7
2	Malignant	Adenocarcinoma	60/M/W	0	30	70	7
3	Malignant	Adenocarcinoma	64/M/W	0	80	20	7
4	Malignant	Adenocarcinoma	68/M/W	0	20	80	6
5	Malignant	Adenocarcinoma	72/M/W	0	30	70	6
6	Malignant	Adenocarcinoma	72/M/W	0	30	70	6
7	Malignant	Adenocarcinoma	78/M/W	0	30	70	7
8	Malignant	Adenocarcinoma	66/M/W	0	30	70	7
9	Malignant	Adenocarcinoma	68/M/W	0	20	80	6
10	Malignant	Adenocarcinoma	52/M/W	0	30	70	7
11	Malignant	Adenocarcinoma	67/M/W	0	20	80	6
12	Malignant	Adenocarcinoma	73/M/W	0	30	70	9
13	Normal	-	51/M/W	0	0	0	
14	Malignant	Adenocarcinoma	69/M/W	0	50	50	6
15	Malignant	Adenocarcinoma	70/M/W	0	60	40	7

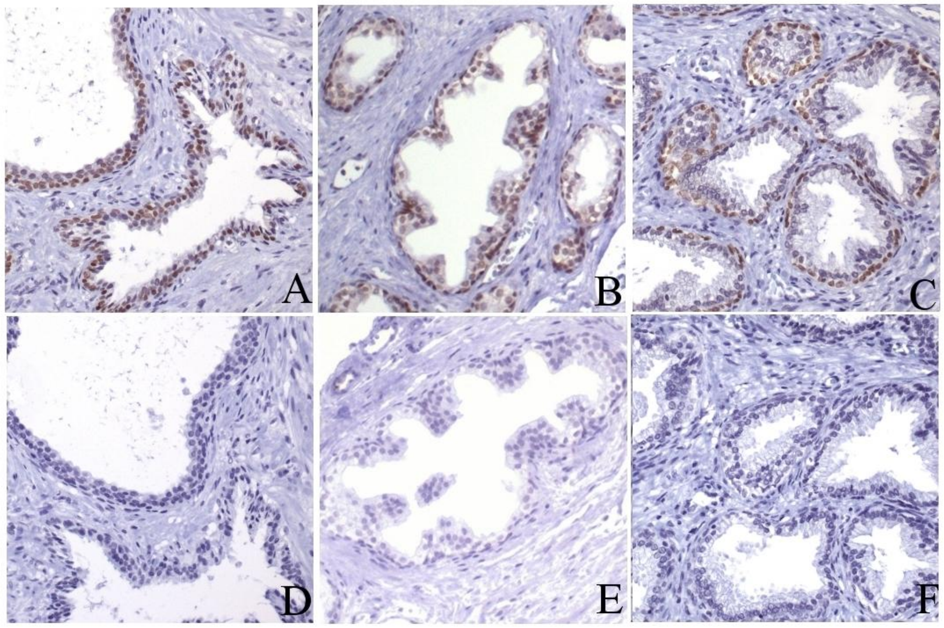
RESULTS

p63 Expression in Benign and Malignant Prostate

As detailed in the Background section, to study the presence and localization of prostate cancer stem cells (CSCs), it is important to include the evaluation of benign glands. p63 expression in benign and malignant prostate was studied since p63 is a prostate basal cell marker (Signoretti *et al,* 2000). p63 was evaluated to discriminate between benign and malignant regions of the prostate, as this protein's expression is limited to basal cells (Signoretti *et al.,* 2000), enabling us to use it for differential diagnosis.

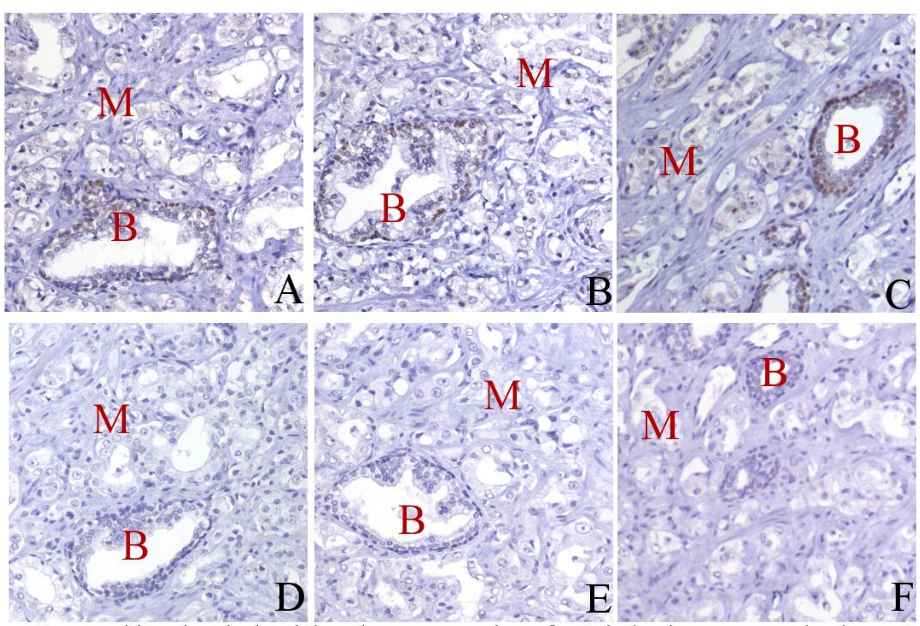
Immunohistochemical analysis of p63 expression in benign and malignant human prostate tissue specimens was performed. Strong p63 reactivity was observed in benign prostate sections stained with monoclonal 4A4 antibody against p63 (**Figure 4A, B, C**). The specificity of the 4A4 antibody was verified when compared to the negative controls stained with mouse IgG (**Figure 4D, E, F**). Nuclear staining in prostate tissue was present in basal cells of the epithelium of benign areas within the pathogenic prostate glands. No expression of p63 was observed in malignant areas of the prostate cancer specimens (**Figure 5**).

Figure 4. p63 Expression in Benign Prostate Glands Analyzed by Immunohistochemistry



Panels A, B, and C depict immunohistochemical expression of p63 in benign prostate glands stained with monoclonal 4A4 (4µg/mL) antibody against p63. Panels D, E, F are negative controls stained with mouse IgG (4µg/mL). Panels A, B, D, E shown at 200X magnification; Panels C and F shown at 100X magnification.

Figure 5. Lack of p63 Expression in Malignant Prostate Cells



Immunohistochemical staining shows expression of p63 in benign prostate glands, and no expression in prostate cancer cells. Panels A, B, and C show benign glands (indicated by B), with p63 expression and malignant regions (indicated by M), lacking p63 expression. Panels D, E, F are negative controls stained with mouse IgG (4µg/mL). All panels shown at 200X magnification.

The p63 results were reproduced and scored using a "+" and "-" system for ten cases, as shown in **Table 4**. The case details for these and all subsequent paraffin-embedded cases were shown in Table 3, in the Materials and Methods section. Strong p63 immunoreactivity was consistently seen in the epithelium of benign prostate glands. p63 expression within these regions is considered specific to cell type, as it is only expressed in benign basal cells, but is not present in surrounding stromal or cancerous cells. Thus, p63 provided a reliable marker to exclude benign prostate cells in future experiments of this MQP.

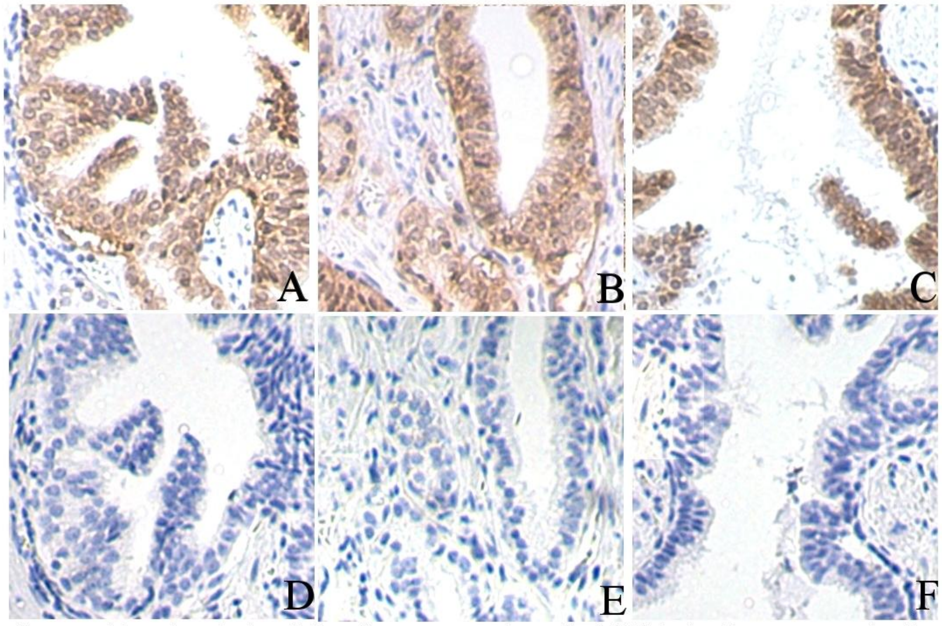
Table 4. Summary of Immunohistochemical Expression of p63 in Benign and Malignant Prostate

Immunohistochemical Expression of p63 in Benign and Malignant Prostate		
	p63	
Case	Benign	Malignant
1	+	-
2	+	-
3	+	-
8	+	-
10	+	-
11	+	-
12	+	-
13	+	-
14	+	-
15	+	-

Trop2 Expression in Benign and Malignant Prostate

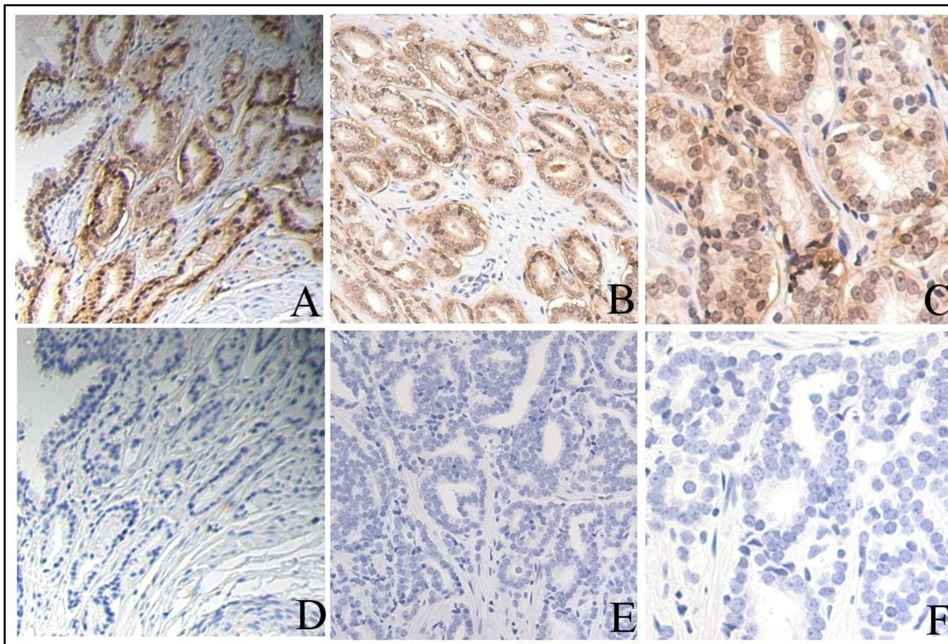
Immunohistochemical analysis of Trop2 expression in benign and malignant human prostate tissue specimens was performed based on published studies showing the potential for Trop2 to be a stem cell candidate (Goldstein *et al.,* 2008). Expression was observed in prostate sections stained with polyclonal antibody against Trop2 (**Figure 6A, B, C**). The specificity of the Trop2 antibody was verified when compared to the negative controls stained with goat IgG (**Figure 6D, E, F**). Trop2 expression was observed in both benign and malignant tissue areas. The staining pattern in malignant areas was very similar to that exhibited in benign glands. Staining was seen in cells of epithelial origin, but not in stromal cells in benign and malignant prostate. In malignant areas of the prostate, staining was diffuse and cytoplasmic (**Figure 7, Panels A, B, C**).

Figure 6. Analysis of Trop2 Expression in Benign Prostate Glands by Immunohistochemistry.



Immunohistochemical staining shows expression of TROP2 in benign prostate glands stained with polyclonal antibody (2.5µg/mL) against TROP2, panels A, B, C. Panels D, E, F are negative controls stained with goat IgG (2.5µg/mL). All panels shown at 200X magnification.

Figure 7. Analysis of Trop2 Expression in Malignant Prostate Cells by Immunohistochemistry.



Immunohistochemical staining shows expression of TROP2 in prostate cancer cells. Panels A, B, and C show malignant regions with TROP2 expression. Panels D, E, and F show negative controls stained with goat IgG. Panels A, B, D, E are shown at 100X magnification; Panels C and F are shown at 200X magnification.

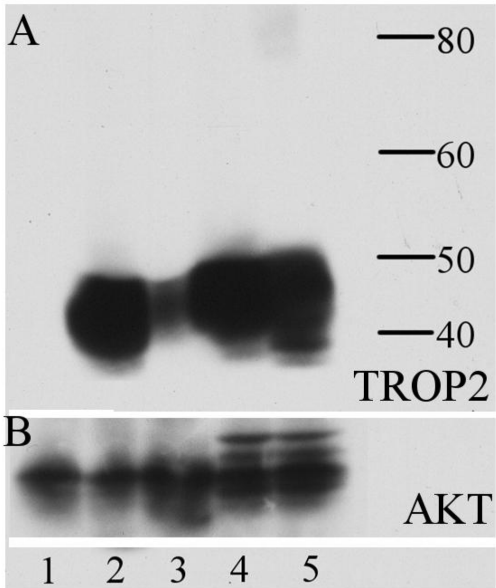
It can be concluded from these results that Trop2 is expressed in both benign and malignant prostate cancer. The IHC results for all the staining performed on paraffin-embedded sections for Trop2 are summarized in **Table 5**. A "+" indicates that staining was visible in both benign and malignant areas, and a "-" indicates that no staining was visible at all. The case details can be seen in Table 3 in the Materials and Methods section.

Table 5. Summary of the Expression of Trop2 in Prostate Tissue Specimens

Expression of Trop2 in Prostate Tissue Specimens	
Case	Trop2
1	-
4	+
5	+
6	+
7	+
8	+
9	+
10	+
13	-
14	+
15	+

Next, immunoblotting analysis of Trop2 expression in benign and prostate tissue, as well as PC3 and PC3-Trop2 cell lysates, was performed to test for protein expression levels, and possible differences between benign and malignant prostate tissue (**Figure 8**). PC3 cells, which do not endogenously express Trop2 were used as a negative control, while the same cell line transfected with Trop2 was used as a positive control. The findings indicate that Trop2 is expressed in both benign and malignant prostate tissue. The difference in expression levels observed within the tissue lysates are not indicative of expression levels as a whole.

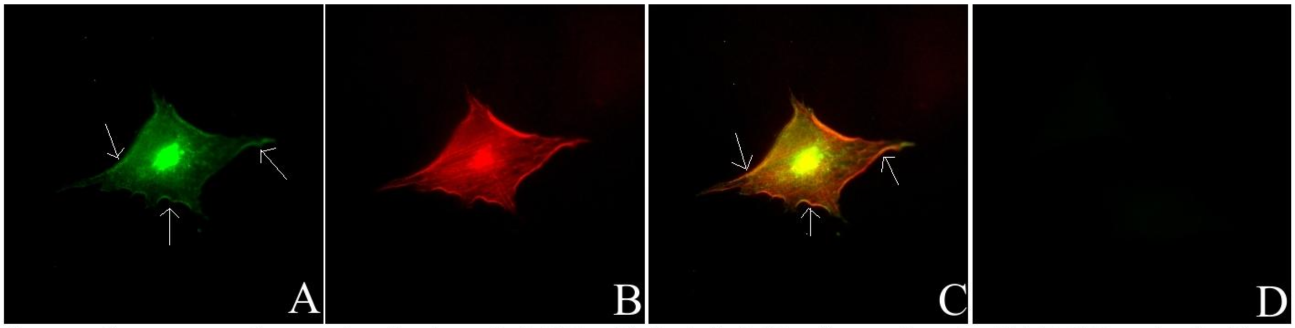
Figure 8. Trop2 Immunoblot.



Immunoblot showing expression of TROP2 in benign and malignant human prostate tissue. Malignant and benign tissue lysates were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis under non-reducing conditions, transferred to a PVDF membrane then immunostained using monoclonal antibody against TROP2. A. The band showing TROP2 expression is confirmed by known molecular weight of 47kDa. L1: PC3 cell lysate; negative control, L2: PC3 cells transfected with TROP2; positive control, L3,4: Malignant Prostate Tissue, L5: Benign Prostate Tissue. B. Loading control was done using an antibody to AKT (molecular weight 65kDA).

Studies have shown that Trop2 expression correlates with poor prognosis in human carcinomas (Goldstein *et al.*, 2008), suggesting that it can play an important role in tumor invasion and migration. Immunofluorescence was performed on the aforementioned PC3 cells seeded on fibronectin to study the expression and localization of Trop2 (**Figure 9A**). Trop2 was found to strongly co-localize with actin in discrete membrane rims (**Fig 9B, C**). Since actin is actively involved in the regulation of cellular movements on substrates, like fibronectin, these results support a role of Trop2 in modulation of PC3 migration.

Figure 9. Trop2 & Actin Immunofluorescence.



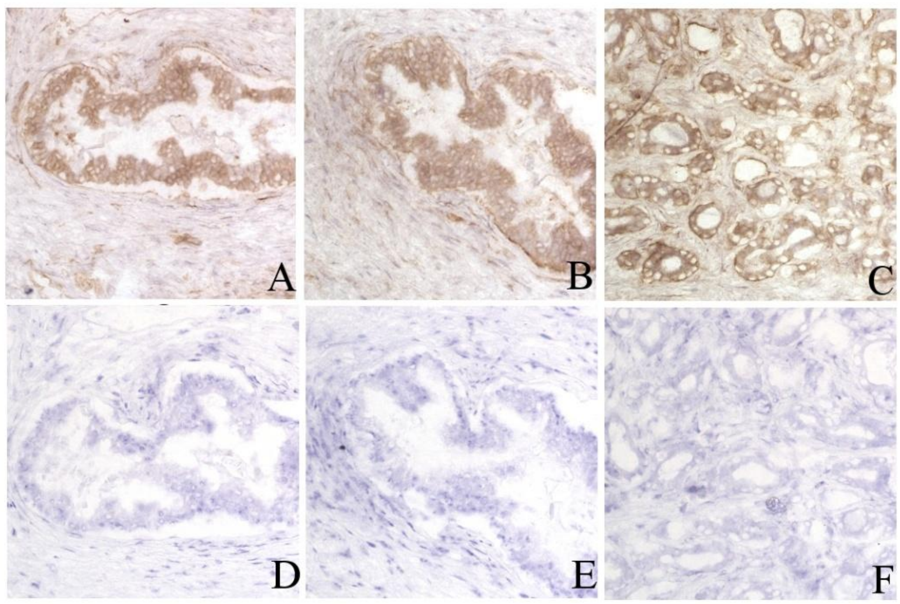
Immunofluorescence shows colocalization of TROP2 with Actin in PC3 cells transfected with TROP2, stained with monoclonal antibody TROP2 T16, panel C. Panel A shows expression of TROP2 in PC3 cells transfected with TROP2. Panel B shows expression of Actin in PC3-TROP2 transfectants. Panel C shows the merge. Panel D shows lack of expression of TROP2 in vector PC3 cells, as the negative control.

Integrin Expression in Frozen Prostate Tissue Specimens

From the aforementioned results, it can be said that Trop2 cannot be used as a cancer stem cell marker, as it shows diffuse expression. According to recent studies, tumor cells expressing α2 integrin and CD133 represent subpopulations with high proliferation potential (Collins *et al.,* 2005). Single α2 IHC was performed as a pre-screen to enable subsequent double staining of α2 and CD133, to isolate cancer stem cells. Immunohistochemistry was performed on paraffin-embedded sections to visualize integrin expression in human prostate tissue (data not shown). However, no expression could be seen for either α2 or β3, as the antibody does not work on paraffin-embedded sections.

IHC was then performed on frozen sections, with successful results only for α2. Expression was observed in frozen prostate sections stained with monoclonal HAS-3 antibody against α2 (**Figure 10, A-C**). The specificity of the α2 antibody was verified when compared to the negative controls stained with mouse IgG (**Figure 10, D-F**). α2 expression is diffuse and can be seen in the epithelial cells of benign glands, as well as in a cancerous region. The expression appears to be at a uniform level throughout the prostate tissue. Our results are also in disagreement with results seen by Nagle *et al.*, as expression levels were the same in both benign prostate and adenocarcinoma.

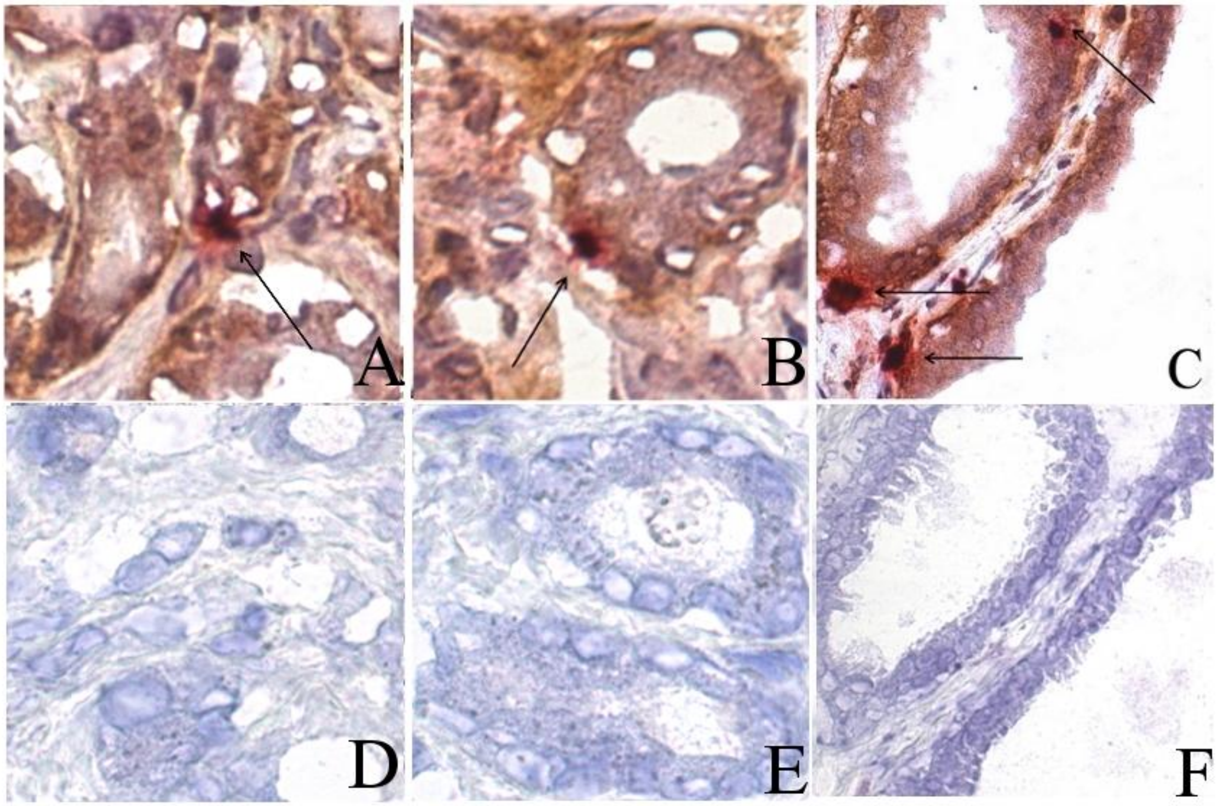
Figure 10. α2 Expression in Frozen Prostate Tissue Analyzed by Immunohistochemistry.



Immunohistochemical staining shows expression of α2 in benign glands and cancerous regions. Panels A-B show benign glands with α2 expression and Panel C shows a malignant area. Panels C-E show negative controls stained with mouse IgG. All panels are shown at 200X magnification.

With successful staining visible on frozen prostate sections, double staining with α2 and CD133 to isolate cancer stem cells was possible. Double staining reveals coexpression of α2 (brown color) and CD133 (red color) within the same prostate tissue section (**Figure 11, A,B,C**). The arrows indicate regions which are stem cell candidates, due to heavy expression of both α2 and CD133 (Gedye *et al.*, 2009).

Figure 11. Double Immunohistochemical Staining to Analyze the Co-Expression of α2 and CD133 in Frozen Prostate Tissue



Double immunohistochemical staining on frozen sections with monoclonal antibody (20μg/mL) HAS-3 against α2 and polyclonal antibody (2.5μg/mL) against CD133 reveals potential stem cells, panels A, B, C (see arrows). Brown color depicts α2 expression, and red color depicts CD133 expression. Panels D, E, and F are negative controls stained with mouse IgG (20μg/mL) and rabbit IgG (2.5μg/mL). All panels shown at 400X magnification.

It cannot be said with certainty whether cancer stem cells were identified, as only 1 of the 6 cases tested showed cancer regions. The double staining revealed cells with high expression of CD133 and α2, characteristics of cells with high clonogenicity (Maitland and Collins, 2008). These cells are potential stem cell candidates, because expression levels are high for both CD133 and α2. Overall, the single staining of α2 was performed on 6 cases, 1 of which had malignant regions. Double staining with CD133, was performed on a total of 6 frozen cases. The IHC results for all the staining performed on frozen sections for CD133 and α2 are summarized in

Table 6. A “+” indicates that staining was visible, and a “-“ indicates that no staining was visible at all.

Table 6. Summary of the Expression of α2 and CD133 in Frozen Prostate Tissue Specimens

Expression of Markers in Frozen Prostate Tissue Specimens			
Case	**α2**		**CD133 & α2 Coexpression**
	Benign	***Malignant***	
16	+		+
17	+		+
18	+		+
19	+		+
20	+		+
21	+	+	+

Black denotes not tested.

β3 Immunohistochemistry

Attempts were made to perform IHC on both paraffin-embedded and frozen sections for β3 integrin. Various methods of antigen retrieval and different antibodies were tested (as per Table 2), but we could not see any positive specific staining for β3 expression, even in known malignant areas (data not shown).

Paraffin-Embedded vs. Frozen Sections

It is important to note that expression for both integrins α2 and β3 could not be detected on paraffin-embedded sections. This lead to the belief that integrin antigens are better preserved in frozen tissue than in their paraffin-embedded counterparts.

DISCUSSION

Treatment against the proliferation of cancer stem cells may well be the next step of cancer therapy. In this MQP, various known and potential markers were analyzed to enable us to potentially isolate prostate cancer stem cells in the future. The hypothesis tested by this MQP was that the existence of an integrin marker for cancer stem cells will co-express with known stem cell markers. We saw that Trop2 and α2 staining was diffuse, thus they could not be used independently as cancer stem cell markers. However, through our observed coexpression of CD133 and α2, we identified stem cells which could be potential cancer stem cells.

The study of p63 expression plays an important role in the study of prostate cancer. An established prostate basal cell marker, p63 is used in the differential diagnosis between benign and malignant areas of the prostate (Signoretti *et al.,* 2000). p63 expression was seen in basal cells of the prostate epithelium which enabled us to identify areas of the prostate tissue specimen that were benign, versus the cancerous regions. It was consistently seen that p63 expression was lacking in malignant areas of the prostate, thus enabling us to conclude that p63 is an excellent negative marker for prostate cancer.

CD133, a known stem cell surface antigen for prostate tissue (Choi *et al.,* 2009), played an important role in this project to identify potential CSCs. As previously suggested, CD133 can be used in combination with other markers to identify CSCs. In contrast, Trop2 was seen to display diffuse expression in both benign prostate epithelium and cancer, thus it cannot be used as a CSC marker.

As previously stated, the focus of this MQP was the characterization of an integrin markers, such as α2 (Maitland and Collins, 2008) or β3 (Goel *et al.,* 2008), for the identification of CSCs. However, it was seen that α2 expression was diffuse, and thus it could not be used to identify CSCs.

Future Experiments

Although in this MQP study we were unable to definitively identify prostate cancer stem cells, we took a clear step forward towards this goal. We established that α2 and Trop2 cannot be used in the identification of CSCs, and that CD133 would need to be studied with another marker to identify CSCs. Future studies would involve isolating CSCs by fluorescence activated cell sorting using CD133 in combination with other markers, and analyzing their potential for multi-lineage differentiation and tumor growth.

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